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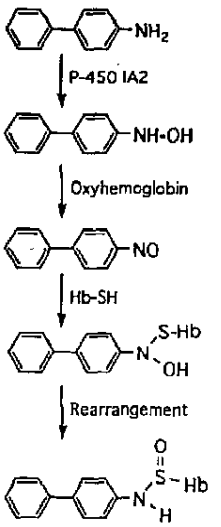
[42] Aromatic Amine–Hemoglobin Adducts

By PAUL L. SKIPPER and W. G. STILLWELL

Human exposure to aromatic amines has multiple documented and potential sources, including tobacco smoke inhalation,[1] workplace contamination,[2] pesticide residues in food,[3] airborne nitroaromatic compounds,[4] and enterobacterial reduction of dyes.[5] Many aromatic amines, perhaps most of the ones with one- and two-ring structures, form hemoglobin adducts (see Scheme I) through a process of hepatic metabolic oxidation to an aryl hydroxylamine followed by heme-mediated oxidation to a nitrosoarene and reaction with a cysteine sulfhydryl group.[6] In human hemoglobin, 4-aminobiphenyl forms a single stable adduct with β93(F9) cysteine, which has been fully characterized as a sulfinic amide.[7] Adduct levels of 4-aminobiphenyl in persons undergoing smoking withdrawal decline nearly as expected on the basis of normal hemoglobin turnover.[8] Other studies with experimental animals, though, indicate that amine adducts or amine-adducted hemoglobin can be removed from circulation more rapidly than normal hemoglobin.[9,10]

[1] M. S. Bryant, P. L. Skipper, S. R. Tannenbaum, and M. Maclure, *Cancer Res.* **47,** 602 (1987).
[2] L. Lewalter and U. Korallus, *Int. Arch. Occup. Environ. Health* **56,** 179 (1985).
[3] G. Sabbioni and H.-G. Neumann, *Carcinogenesis (London)* **11,** 111 (1990).
[4] J. Arey, B. Zielinska, R. Atkinson, and A. M. Winer, *Atmos. Environ.* **21,** 1437 (1987).
[5] B. W. Manning, C. E. Cerniglia, and T. W. Federle, *Appl. Environ. Microbiol.* **50,** 10 (1985).
[6] H.-G. Neumann, *in* "Molecular Dosimetry and Human Cancer" (J. D. Groopman and P. L. Skipper, eds.), p. 363. CRC Press, Boca Raton, FL, 1991.
[7] D. Ringe, R. J. Turesky, P. L. Skipper, and S. R. Tannenbaum, *Chem. Res. Toxicol.* **1,** 22 (1988).
[8] M. Maclure, M. S. Bryant, P. L. Skipper, and S. R. Tannenbaum, *Cancer Res.* **50,** 181 (1990).
[9] H.-G. Neumann, *IARC Sci. Publ.* **59,** 115 (1984).
[10] K. L. Cheever, D. E. Richards, W. W. Weigel, K. B. Begley, D. G. DeBord, T. F. Swearengin, and R. E. Savage, Jr., *Fundam. Appl. Toxicol.* **14,** 273 (1990).



SCHEME I

Overall conversion of aromatic amines to adducts can be very high, as much as 10% of intake, making these compounds very amenable to biomonitoring. The adducts are readily hydrolyzed to regenerate free aromatic amines, which can be determined by gas chromatographic analysis.[1,11] The most sensitive and selective detection, which is necessary for studies such as those involving tobacco smoke exposure, is negative-ion chemical ionization mass spectrometry, and this technique is described in detail in [41]. Other exposure scenarios might not demand as sophisticated and expensive equipment, and the interested researcher should consider if alternatives such as electron capture or nitrogen-specific detection would be satisfactory.

Many of the recommendations and procedures that are described below are based on the assumption that the studies will involve extremely low levels of adduct, on the order of 0.01–1 pmol/g hemoglobin. The degree of stringency can undoubtedly be reduced if higher adduct levels are involved. However, it cannot be overemphasized that appropriate controls be employed: the determination of adducts is through analysis of aromatic amines, which are no different from amines that produced the adduct in the first place, and if these are significant environmental contaminants,

[11] L. C. Green, P. L. Skipper, R. J. Turesky, M. S. Bryant, and S. R. Tannenbaum, *Cancer Res.* **44**, 4254 (1984).

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then the results can be compromised if the environmental contamination extends to the analytical equipment or laboratory.

Reagents and Solvents

Hexane of a grade suitable for organic trace analysis such as Baker Resi-analyzed (Phillipsburg, NJ) is distilled sequentially through two high efficiency fractionating stills. An effective column for the stills can be made by filling a 50-cm jacketed Vigreux column with small glass helices. Water is distilled from a dilute $KMnO_4$/KOH solution. Trimethylamine solution in hexane is prepared by adding 1 ml 10 *M* NaOH to an aqueous solution of 1 g trimethylamine hydrochloride and extracting with 5 ml hexane. It should be stored in a tightly stoppered bottle and not kept more than 1 week. Derivatization grade pentafluoropropionic anhydride (PFPA) should be used.

Equipment

Ideally, all equipment should be dedicated. This applies especially to items such as rotary evaporators and associated glassware, stills, and dialysis equipment. All glassware should be cleaned by soaking in a bath of ethanolic (200 proof) KOH and then rinsed with distilled H_2O and oven dried.

Isolation of Hemoglobin

Red blood cells are separated from whole blood (10 ml) by centrifugation and washed three times with phosphate-buffered normal saline (PBS). They may then be used directly or frozen for storage. Analysis after as long as 2 years in storage at $-20°$ has indicated that there is no loss of adducts over this length of time. Freezing is also convenient in that it facilitates cell lysis, which is accomplished by adding 2–3 volumes of distilled H_2O per volume of packed red blood cells and 2 ml toluene, which has previously been extracted with 1 *N* HCl. The mixture is shaken vigorously and allowed to stand for 30 min, after which it is centrifuged at 10,000 g for 20 min. The aqueous phase is then dialyzed against distilled water for 3 days, the water being changed each day.

Adduct Hydrolysis

The dialyzed hemoglobin solutions are transferred to 50-ml screw-cap tared centrifuge tubes and the weight of each is determined. The

hemoglobin concentration is determined by Drabkin's assay. Appropriate internal standards are added with complete mixing and the solutions are then allowed to stand for ½ to 1 hr. Following this a solution of 10 *M* NaOH is added, using 0.01 volume per volume of hemoglobin solution. The color of the solution should change from red to brown rather quickly. If the hemoglobin concentrations are particularly high (>50 mg/ml), more NaOH may be needed. After the addition of NaOH, the solutions are held for 2–3 hr at room temperature.

Extraction of the amines is performed twice with a volume of hexane sufficient to nearly fill the centrifuge tube. The tubes are shaken gently for several minutes and then frozen to break the resultant emulsion. Too vigorous extraction results in the formation of unbreakable emulsions. The hexane layer is removed by pipette and passed through a column of drying agents (Na_2SO_4 and $MgSO_4$) into a 50-ml pear-shaped flask. A convenient column can be made from Kimble disposable serological 10-ml glass pipettes as follows. First, a glass bead with diameter slightly greater than the pipette opening is introduced into the pipette. Anhydrous Na_2SO_4 is then added to form a layer 2 cm deep. Finally, anhydrous $MgSO_4$ is added to form an upper layer of about 2 cm. The Na_2SO_4, being granular, serves to facilitate flow around the glass bead while blocking the passage of any of the powdery $MgSO_4$ into the receiving flask.

We have found hexane to be the optimum solvent for amine extractions because it extracts so little else from the hydrolyzates. It is clearly suitable for the relatively nonpolar unsubstituted or alkyl-substituted aromatic monoamines. Other have also used hexane for extraction of chlorinated anilines.[12] One laboratory, though, has found hexane to be ineffective and uses dichloromethane instead.[13]

Derivatization

The dried, combined hexane extracts are treated with 5–10 μl of trimethylamine in hexane and 3–5 μl of PFPA. After 10 min or longer the solvent is removed with a rotary evaporator until the volume has been reduced to 1–2 ml. The solution is transferred to a ½ dram vial and the remainder of the solvent is removed. This can be performed conveniently with a rotary evaporator using a container for the vial made from a standard 24/40 ground glass outer joint. These joints are supplied with an integral length of glass tubing that has a suitable inside diameter to contain the

[12] G. Sabbioni, *Chem.-Biol. Interact.* **81**, 91 (1992).
[13] P. DelSanto, G. Moneti, M. Salvadori, C. Saltutti, A. DelleRose, and P. Dolara, *Cancer Lett.* **60**, 245 (1991).

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vials. The tubing is cut to an appropriate length, and the end is flame-sealed. When analyzing particularly volatile amines, such as aniline or alkylanilines, it is important not to leave the vials on the evaporator any longer than necessary to remove the hexane. The samples are reconstituted in a minimal volume, typically 20 μl, of hexane or heptane for injection onto the capillary GC column. A Teflon liner must be used in the caps of these vials because the rubber liners leach contaminants that interfere with the analysis.

Internal Standards

The best internal standard is hemoglobin that has been adducted by the amine of interest labeled with a stable isotope. Adducted hemoglobin can be made by reacting freshly isolated human hemoglobin with the appropriate aromatic hydroxylamine. Details of the synthesis of the hydroxylamines will vary depending on the structure, but in general they can be formed by reduction of the corresponding nitroarenes. After reaction of hemoglobin with the aromatic hydroxylamine, the mixture is dialyzed to remove unbound material. It can then be diluted to a suitable concentration of adduct, divided into aliquots sufficient for a batch of samples to be analyzed, and stored frozen at −20°. The concentration of adduct is determined by using the normal isotope form of the amine as an internal standard.

An alternative but less satisfactory internal standard is isotopically labeled amine, which is prepared as a 0.1 *N* HCl solution. Most aromatic amines are stable in acidic aqueous solution, so these can be stored in the refrigerator for extended periods of time.

Isotopically labeled internal standards are, of course, unsuitable unless mass selective detection is used. If it is desired to use an electron capture or flame ionization detector, then monofluoroaromatic amines or amine adducts make good internal standards. The substitution of fluorine for one hydrogen atom has only a slight effect on the physical properties of the amine, just enough to render it separable by capillary GC from the unsubstituted amine.

Chromatographic Analysis

Gas chromatography is best performed using high-resolution bonded stationary-phase capillary columns. When using stable isotope-labeled internal standards and mass spectrometric detection, nonpolar stationary phases such as DB-1 (J & W Scientific, Folsom, CA) are to be preferred because they are extremely durable and performance changes little with

time. Separation of monofluoro internal standards, however, requires a polar stationary phase such as Supelcowax 10 (Supelco, Bellefonte, PA). These phases are far more temperature sensitive and degrade slowly when heated to the temperatures required to elute naphthyl- and biphenylamines, so care must be taken in their use.

Simple temperature programs are usually sufficient to achieve good resolution of almost all aromatic amines in one run. The sample can be injected at 60°–100°. Temperature is then ramped linearly at 15°–20°/min to the chosen final temperature. A postrun temperature hold of 2–5 min at a temperature 20° higher than that required to elute the last amine of interest is advisable to avoid carryover from one run to the next.

Splitless injection is essential in the analysis of trace levels of adducts, such as those produced by tobacco smoke exposure, in order to obtain high sample loading of capillary columns. On-column injection is an alternative that achieves even higher sample loading. In our 4-aminobiphenyl adduct studies we routinely inject 20% of the entire sample (4 μl out of 20 μl) in the splitless mode.

Mass Spectrometry

This section will only deal with some specifics that are not addressed in the more general discussion of [41]. It is assumed that the mass spectrometer is equipped for negative-ion chemical ionization.

The PFPA derivatives of aromatic amines produce a very simple negative-ion mass spectrum[14] generally composed of a very weak ion at 1 mass unit less than the molecular weight, the base peak produced by loss of the elements of HF, and several other weak ions, including one at 38 mass units less than the molecular weight, probably resulting from loss of F_2. Each amine is detected by monitoring the ion current at the mass/charge ratio corresponding to the mass of the base peak, which is 20 mass units less than the molecular weight. It should be realized that a monofluoramine will produce significant ion current at 38 mass units below its molecular weight, which is also the same value as the monitored base peak ion produced by the nonfluorinated amine. Thus, if a monofluoramine is used as internal standard, it is necessary that a GC column be used that can separate the two amines.

[14] W. G. Stillwell, M. S. Bryant, and J. S. Wishnok, *Biomed. Environ. Mass Spectrom.* **14**, 221 (1987).

There is no significant mass defect for most of the PFPA derivatives. Thus, the m/z values that are monitored should be adjusted only for the offset observed in the mass axis calibration.

We have not observed any significant difference in mass spectrometric detector response to the pairs of isomers 4-aminobiphenyl and 4-aminobiphenyl-d_9 or aniline and aniline-d_5. The unadjusted peak area ratio for quantitation is therefore applicable, but it is recommended that this be verified for a given instrument or if other isotopes such as ^{13}C or ^{15}N are used.

Comments

It is critically important in these analyses to maintain cleanliness of materials and workplace and to ensure that samples contact only inert materials. This advice is pertinent, of course, to all trace level analysis, but is repeated here because it is easy to overlook if one is used to part per million levels rather than part per trillion, and one part per trillion is in fact possible with the techniques described in this chapter.

Rubber is one of the most significant potential sources of contamination. We can routinely find dibenzylamine in samples, and it is possible that this amine comes from the rubber stoppers used in blood collection tubes.[15] We have also detected other aromatic amines with antioxidant-type structure and suspect that these too arise from contact with rubber at some point. Thus, every effort should be made to use only glass, stainless steel, or Teflon materials.

The precision of this assay can be such that a coefficient of variation (CV) of 5–10% can be obtained. When analyzing for 4-aminobiphenyl adducts with 4′-fluoro-4-aminobiphenyl as standard we obtain a CV near 10%. This is reduced to near 5% when hemoglobin adducted with perdeutero 4-ABP is used. The use of an amine with a different structure can lead to a considerably greater CV.

Acknowledgment

This work was supported by grants from NIEHS (Nos. ES04675 and ES02109).

[15] J. W. Danielson, G. S. Oxborrow, and A. M. Placencia, *J. Parenter. Sci. Technol.* **38**, 90 (1984).